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Jeffrey W. Cary <sup>a</sup>; Ana M. Calvo <sup>b</sup>

<sup>a</sup> Food and Feed Safety Research Unit, Agricultural Research Service, U.S. Department of Agriculture, New Orleans, Louisiana, United States <sup>b</sup> Department of Biological Science, Northern Illinois University, DeKalb, Illinois, United States

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## REGULATION OF *ASPERGILLUS* MYCOTOXIN BIOSYNTHESIS

**JEFFREY W. CARY**

Food and Feed Safety Research Unit, Agricultural Research Service,  
U.S. Department of Agriculture, New Orleans, Louisiana, United States

**ANA M. CALVO**

Department of Biological Science, Northern Illinois University, DeKalb,  
Illinois, United States

*The genus Aspergillus produces a number of mycotoxins that pose adverse economic and health impacts on humans and animals. These include the toxic and carcinogenic polyketide-derived mycotoxin, sterigmatocystin, and aflatoxins, produced by Aspergillus nidulans and Aspergillus flavus, respectively. Studies have identified the gene clusters that are responsible for the synthesis of these toxins; however, there is still much to be elucidated regarding the signal transduction pathways and globally acting regulators that control production of these toxins during fungal contamination of crops. In many cases, the mechanisms by which the fungus responds to environmental and plant-based factors have been found to not only control toxin production but also fungal growth and development.*

**Keywords** aflatoxins, sterigmatocystin, mycotoxins, *Aspergillus flavus*, *Aspergillus nidulans*, gene cluster, transcription factor, secondary metabolite

### Introduction

Some saprophytic species of the genus *Aspergillus* have the ability to colonize agricultural crops of economic importance and produce highly toxic and carcinogenic mycotoxins. A few of these toxigenic species are also common opportunistic pathogens causing aspergillosis in both humans and animals. Elucidation of the genetic mechanisms regulating mycotoxin biosynthesis in these *Aspergilli* may provide the basic knowledge needed to develop

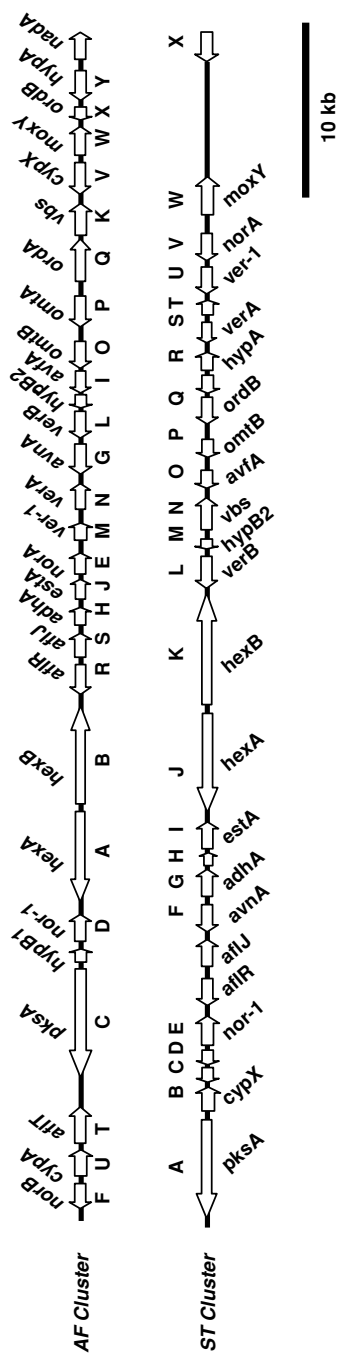
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Address correspondence to J. W. Cary, USDA, ARS, Southern Regional Research Center, New Orleans, LA, 70124, USA. E-mail: Jeff.Cary@ars.usda.gov

strategies for the control of the devastating effects that mycotoxins have on both public health and agricultural economics. In the last few years our understanding of the regulatory mechanisms in these fungi has increased considerably. This review will focus mainly on studies in the three *Aspergillus* species where the majority of this progress has been achieved: *Aspergillus flavus* and *A. parasiticus*, producers of aflatoxins (AF), the most toxic and carcinogenic mycotoxins known, and the model fungus *Aspergillus nidulans*, producer of sterigmatocystin (ST) toxin, the penultimate precursor in the AF pathway.

### Aflatoxin Pathway Specific Regulators

The AF biosynthetic genes found in *A. flavus* and *A. parasiticus* consist of 28 known genes that are clustered in a region spanning approximately 70 kb (Figure 1) (Bhatnagar et al., 2003; Yu et al., 2004; Ehrlich, Yu, and Cotty, 2005). In addition to the genes that encode enzymes needed for AF biosynthesis the cluster also harbors genes required for transcriptional activation of the biosynthetic genes. A positive regulatory gene, *aflR*, encodes a sequence-specific, Gal4-type C6-zinc binuclear cluster DNA binding protein that is required for transcriptional activation of the AF structural genes (Chang et al., 1993; Woloshuk et al., 1994). *AflR* also regulates the expression of *A. nidulans* ST biosynthetic genes that are also organized in a cluster together with the *aflR* gene (Fernandes et al., 1998). The *aflR* protein binds to the palindromic sequence 5'-TCGN<sub>3</sub>CGA-3' within the promoter region of AF structural genes (Fernandes et al., 1998; Ehrlich, Montalbano, Cary, 1999). The *nadA* gene, located adjacent to the *A. flavus parasiticus* AF gene cluster, was thought to be part of a sugar utilization gene cluster (Yu et al., 2000). However, recent evidence demonstrated that *nadA* is involved in the formation of G aflatoxins (Cai et al., 2008) and its expression was under the control of *aflR* (Price et al., 2006). Interestingly, a study by Price and colleagues (2006) using *A. flavus* expressed sequence tag (EST) microarray transcription profiling studies identified two additional genes (*niiA* and *hlyC*) located well outside of the AF gene cluster that were upregulated under AF-conductive conditions. In these genes, the consensus *aflR* binding sites were observed; however, they were located from 1.8 to 2.3 kb upstream



**FIGURE 1** Comparison of the AF and ST gene clusters. Genomic nomenclature at top of AF cluster and below ST cluster represents functionality of genes while the single letter nomenclature is also presented. Comparative information on the AF and ST gene clusters can be obtained in Ehrlich, Yu, and Cotty (2005).

of the translational start sites. Often there is more than one *aflR* consensus binding site within the promoter region of a pathway gene, as in the case of the *aflG* (*avnA*) gene. In this gene, the binding site closest to the transcriptional start point (tsp) is required for transcriptional activation (Cary et al., 2000). However, in the case of the *aflC* (*pksA*) gene, where two *aflR* binding sites are located within about 400 bp of the transcriptional start point (tsp), both sites have been found to contribute to *aflC* promoter activity (Ehrlich et al., 2002). Although the *aflR* promoter does not contain a consensus *aflR* binding site, it was shown by electrophoretic mobility shift assays (EMSA) to bind a recombinant form of *aflR* protein; thus *aflR* may be self-regulating (Ehrlich et al., 1999). A number of studies have also shown that elements upstream of the *aflR* coding region may serve as binding sites for proteins that negatively regulate *aflR* expression (Chang et al., 1999; Ehrlich et al., 1999; Chang et al., 2000). Additionally, loss of *aflR* does not result in loss of spore or sclerotial formation; however, it does impact the numbers of spores or sclerotia being formed (Chang et al., 2002). In *A. parasiticus* a second copy of a nonfunctional *aflR* protein (*aflR-2*) was shown to be able to bind to the AF promoters with a consensus *aflR* binding site *in vivo*, and a regulatory mechanism based on the competition of both *aflR* forms has been suggested (Roze et al., 2007).

Another gene that appears to play a role in the regulation of AF production is *aflJ* (new designation *aflS*). *AflJ* does not demonstrate any significant homology to other genes or deduced protein sequences present in the genome databases (Meyers et al., 1998). It is adjacent to and divergently transcribed from *aflR*. Although the exact function of *aflJ* has not been determined, it is required for production of wild-type levels of AF and it has been shown to interact with *aflR* (Meyers et al., 1998; Chang, 2003). *aflJ* transcription is regulated by *AflR* as overexpression of *aflR* resulted in increased *aflJ* transcription (Du et al., 2007). Interestingly, although *aflJ* overexpression increased AF production, its overexpression did not result in elevated transcription of mid- to late-AF pathway intermediate genes such as *aflM* (*ver-1*) or *aflP* (*omtA*). However, transformants expressing *aflR* and *aflJ* produced five times more *aflC* transcripts and four times more *aflD* (*nor-1*) transcripts than strains expressing only *aflR* (Du et al.,

2007). This would suggest that *aflJ* modulates the regulation of early genes in the AF biosynthetic pathway. Loss of *aflR* or *aflJ* does not result in loss of spore or sclerotial formation; however, it does impact sclerotial morphology (Chang et al., 2002). The koji mold *A. sojae*, used in food fermentations, does not produce AF despite having a complete AF gene cluster. The inability of the fungus to produce AF is due to a mutation in *aflR* that results in a pretermination defect causing truncation of the carboxyl terminus of the predicted protein. This results in *aflR* not being able to interact with AflJ as determined in yeast two-hybrid assays (Chang, 2004).

Another gene encoding a transcription activation factor regulating mycotoxin production is *gliZ*, found in opportunistic human and animal pathogenic strains of *Aspergillus fumigatus*. GliZ, encoding a putative Zn(2)Cys(6) binuclear transcription factor, activates the gliotoxin gene cluster (Bok, Chung, et al., 2006). Deletion of *gliZ* resulted in loss of gliotoxin production but did not significantly alter the virulence of the fungus in a murine model, indicating that other metabolites in addition to gliotoxin are involved in virulence in *A. fumigatus* (Bok, Chung, et al., 2006).

## Effect of Environmental Factors on AF Production and Fungal Development

### *Effect of Carbon and Nitrogen Source and pH*

In addition to pathway-specific regulators, production of AF is also under the control a number of global regulatory networks that respond to environmental and nutritional cues. These include responses to nutritional factors such as carbon and nitrogen sources and environmental factors such as pH, light, oxidative stress, and temperature. Globally acting regulatory proteins such as AreA involved in nitrogen signaling, CreA involved in carbon signaling, and PacC involved in pH mediated signaling either positively or negatively influence AF production.

Nitrogen source plays an important role in AF biosynthesis (Bhatnagar et al., 1986). In general, nitrate inhibits AF production, while ammonium salts are conducive. However, nitrate has

been shown to enhance ST production in *A. nidulans*, while ammonium-based media repressed ST (Feng and Leonard, 1998). Nitrogen metabolism in fungi is regulated by the globally acting transcription factor AreA (Marzluf, 1997). Overexpression of the *aflR* gene in *A. parasiticus* resulted in release of nitrate inhibition on AF biosynthesis indicating that AreA imparts its control on toxin synthesis either directly or indirectly via *aflR* (Chang et al., 1995). Electrophoretic mobility shift assays (EMSA) indicated that the *A. parasiticus* AreA binds within the *aflR-aflJ* intergenic region and a number of putative AreA GATA binding sites are present within this region (Chang et al., 2000). Analysis of the effects of nitrate on AF production and expression of *aflR* and *aflJ* in a number of *Aspergillus flavus* strains indicated variability in nitrogen regulation and this variability could often be found to correspond to differences in the number of GATA sites near the *aflJ* tsp (Ehrlich and Cotty, 2002). Nitrogen source can also influence formation of sclerotia in *A. flavus*. Studies of *A. flavus* growth on agar media containing either nitrate or ammonium as the sole nitrogen source showed that sclerotial development occurred with nitrate but not with ammonium (Bennett et al., 1979).

AF biosynthesis is induced by simple sugars, such as glucose and sucrose, that are present or generated by fungal hydrolytic enzymes during invasion of seed tissues (Buchanan and Lewis, 1984; Woloshuk et al., 1997). There is no evidence for the involvement of carbon catabolite repression by CreA in regulation of AF production, as in most cases glucose stimulates AF production. No putative CreA binding sites have been identified in the promoters of AF pathway genes. Carbon catabolite repression by CreA is mediated via signal transduction that utilizes cAMP as a second messenger. Internal cAMP levels have been shown to play a role in induction of AF biosynthesis (Roze et al., 2004). A novel cAMP-response element, Cre1, was identified in the promoter of the *aflD* gene and when this site was mutated there was a threefold decrease in cAMP-mediated *aflD* promoter activity under AF-conductive growth conditions (Roze, Miller, et al., 2004). South-western blotting identified a 32 kDa protein (p32) that specifically bound the *aflD* promoter and was co-immunoprecipitated by anti-*aflR* antibody. It was hypothesized that p32 assists AflR in binding to the *aflD* promoter, thereby modulating *aflD* expression in response to environmental cues (Roze, Miller, et al., 2004).

AF and ST production, in general, is greatest in acidic medium and tends to decrease as the pH of the medium increases (Keller et al., 1997). An atypical, West African strain of *A. flavus* was identified that produced less AF in acidic medium (Ehrlich et al., 2005). The strain is designated *A. flavus* S<sub>BG</sub> as it produced both AFB<sub>1</sub> and AFG<sub>1</sub> whereas most *A. flavus* strains only produce the B AFs. Interestingly, the changes in AF production between the S<sub>B</sub> and S<sub>BG</sub> strains did not correlate well with changes in *aflR* expression, indicating that pH may be exerting its effects on other cellular metabolic processes that in turn regulate AF biosynthesis. Response to changes in pH is regulated by the globally acting transcription factor PacC, which is posttranslationally modified by a pH-sensing protease (Tilburn et al., 1995). A number of putative PacC binding sites have been identified in the promoters of AF biosynthetic genes and could be involved in negatively regulating AF biosynthesis during growth at alkaline pH (Ehrlich et al., 1999; Ehrlich et al., 2002). Fungal development also appears to respond to changes in pH as sclerotial production was found to be reduced by 50% at pH 4.0 or less while AF production was at its maximal (Cotty, 1988).

### *Effect of Plant Metabolites*

A number of plant-based metabolites have been shown to reduce AF production as well as alter fungal development (reviewed in Holmes et al., 2008). These include volatile aldehydes (Zeringue and McCormick, 1990; Greene-McDowelle et al., 1999; Wright et al., 2000), flavanoid compounds (Norton, 1999), neem leaf extracts, and jasmonic acid (Bhatnagar and McCormick, 1988; Goodrich-Tanrikulu et al., 1995). Volatile aldehydes, jasmonic acid, and methyl jasmonate are all biologically active end products of the LOX pathways in plants. LOX pathway metabolic precursors (i.e., oxylipins) have been shown have significant effects on fungal development and toxin production (see below). These effects are more than likely mediated by the above-described end products of the LOX pathways that can function as signaling molecules in transduction pathways that regulate a number of biological processes (Feussner and Wasternack, 2002). Ethylene and CO<sub>2</sub> treatment have been shown to reduce AF production in *A. parasiticus*. Treatment with ethylene reduced AF accumulation in a

dose-dependent manner, with a tenfold reduction observed when *A. parasiticus* was treated with 146 ppm ethylene. CO<sub>2</sub> at 0.1% also reduced AF accumulation about fivefold; however, adding more CO<sub>2</sub> (0.7% or 3.0%) reversed the inhibitory effect (Roze, Calvo, et al., 2004). Ethylene decreased the level of transcription of the *aflD* gene in *A. parasiticus*, suggesting that ethylene acts directly or indirectly on AF promoter function. Treatment of the fungus with 1-methyl-cyclopropene (MCP), an inhibitor of the ethylene response in plants, reversed the inhibitory effect of ethylene, prompting the hypothesis that ethylene affects toxin synthesis via an ethylene receptor that functions as part of a signal transduction pathway. In this same study, treatment of peanut seeds with 2 ppm ethylene and/or two concentrations of CO<sub>2</sub> (0.05% or 0.1%) inhibited AF accumulation up to fivefold. This study was followed by a more in-depth study on the effects of ethylene and CO<sub>2</sub> concentrations on stored peanut seed (Gunterus et al., 2007). The authors found that the greatest effect on AFB1 accumulation was obtained at 100 ppm ethylene on peanut that had been inoculated with *A. parasiticus* and grown for 5 days before determining AF levels. Treatment with CO<sub>2</sub> alone (0.1%) generated a similar inhibitory effect on AF accumulation, as did 100 ppm ethylene; however, co-treatment of peanuts with both ethylene (either 2 or 100 ppm) and CO<sub>2</sub> (0.1%) did not show a statistically significant inhibitory effect.

Another volatile compound described to affect AF production is 2-ethyl-1-hexanol. 2-buten-1-ol. 2-ethyl-1-hexanol stimulates AF production in *A. parasiticus* (Roze et al., 2007). However, 2-buten-1-ol showed a dose-dependent up-regulatory or down-regulatory effect not only on AF gene transcription and AF accumulation, but also on production of asexual spores (Roze et al., 2007). Both 2-ethyl-1-hexanol and 2-buten-1-ol were found to be produced by *A. parasiticus* and at a higher level by *A. nidulans*.

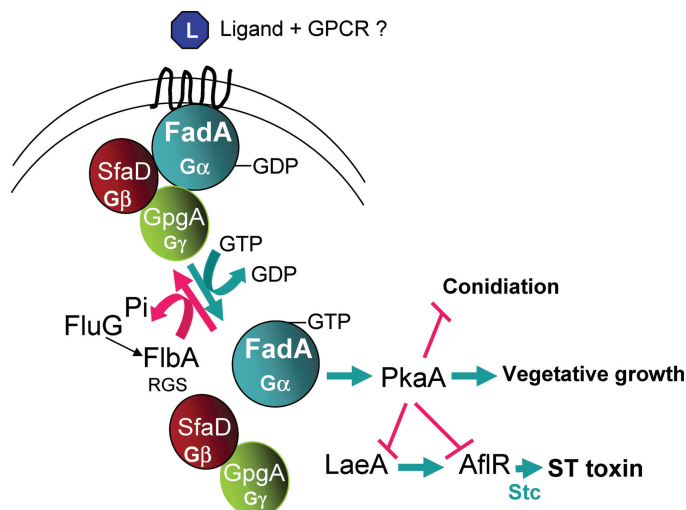
One of the most in-depth areas of study on the molecular genetics of fungal responses to plant metabolites is the group of metabolites known collectively as oxylipins. Oxylipins are hormone-like molecules that have been implicated as signaling molecules for cross-kingdom communication in plant-pathogen interactions (Oh et al., 2001). Oxylipins are generated as a result of the hydroxylation of C18 unsaturated fatty acids (Liavonchanka and Feussner, 2006). In plants, linoleic

(18:2) and linolenic acid (18:3) can be converted by the action of LOX enzymes to 13S-hydroperoxy linoleic acid (13S-HPODE) and 9S-hydroperoxy linoleic acid (9S-HPODE). These plant oxylipins closely resemble the fatty acid-derived compounds known as psi factors, produced by aspergilli, that can also influence fungal development and toxin production (Champe et al., 1987). Both *A. flavus* and *A. nidulans* development is affected by the presence of 13S-HPODE and 9S-HPODE in a dose-dependent manner (Calvo et al., 1999). Addition of pure 13S-HPODE to cultures of either *A. parasiticus* or *A. nidulans* was shown to repress AF and ST production, respectively, while pure 9S-HPODE increased toxin production (Burow et al., 1997). Interestingly, it appears that growth of the fungus on natural substrates, such as maize or peanut seed, results in altered expression of seed LOX genes, thus leading to changes in the levels of plant oxylipins. The maize 9-LOX gene was up-regulated upon infection with *A. flavus* (Wilson et al., 2001) while the peanut 13-LOX genes were down-regulated (Tsitsigiannis et al., 2005). Postulating that plant oxylipins mimic or interfere with biological activities of endogenous fungal oxylipins, Brodhagen and colleagues (2008) looked at the ability of a maize oxylipin biosynthetic gene (*ZmLOX3*) to substitute functionally for *A. nidulans* *ppo* genes that encode dioxygenases involved in synthesis of fungal psi factors. The maize *ZmLOX3* gene was introduced into wild-type *A. nidulans* and a  $\Delta ppoAC$  strain (reduced in production of oxylipins psiB $\alpha$  and psiB $\beta$ , conidia, and ST). Increased production of conidia and ST was observed in both strains. It was also observed that peanut seed *pnlox2-3* expression was decreased upon infection by *A. nidulans*  $\Delta ppo$  mutants compared with levels expressed upon infection by a wild-type strain. These two experiments suggest that oxylipin cross-talk in the host seed-*Aspergillus* interaction may be reciprocal.

## Global Regulation of Mycotoxin Production

### *FadA* Signaling Pathway

Fungal growth, development, and secondary metabolism are genetically associated by signaling pathways, including G-protein signal transduction pathways. This topic has been covered



**FIGURE 2** FadA signal transduction pathway connecting development and production of sterigmatocystin production in *A. nidulans*. A yet unknown ligand initiates the activation of FadA by dissociation from the G $\beta$  and G $\gamma$  subunits. In this form FadA activates PkaA, which leads to vegetative growth and to inhibition of conidiation and mycotoxin production.

extensively in previous reviews (Calvo et al., 2002; Yu and Keller, 2005; Brodhagen and Keller, 2006). In general, G-protein-coupled receptors activated by a ligand transmit the signal to two downstream signaling pathways, a G-protein-cAMP-dependent kinase (Yu and Keller, 2005) and/or mitogen-activated protein kinase pathway, affecting several cell functions, including morphogenesis and mycotoxin biosynthesis (Paoletti et al., 2007; Atoui et al., 2008). The *A. nidulans* FadA pathway is a well-characterized example of a G-protein-cAMP-dependent kinase signaling pathway (Figure 2) (Hicks et al., 1997; Shimizu and Keller, 2001). In this signaling pathway a protein, designated FluG, is involved in the synthesis of a small, diffusible signal molecule participating in the positive regulation of FlbA (Lee and Adams, 1994, 1996; Seo et al., 2003), a GTPase-activating protein that negatively regulates FadA ( $\alpha$ -subunit of the heterotrimeric G-protein). Activation of FadA results in its dissociation from the G $\beta$ -G $\gamma$  subunits and it in turn activates expression of a cAMP-dependent kinase, PkaA, inducing vegetative growth while inhibiting conidiation, sexual development, and ST production. PkaA represses ST production

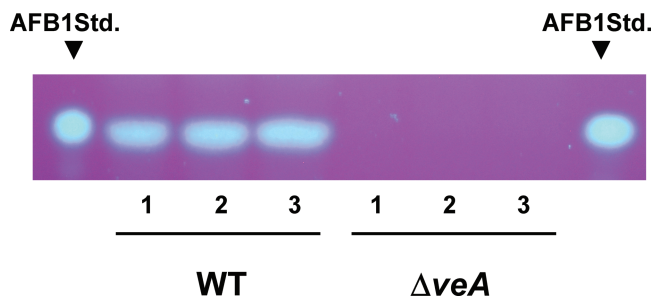
by both inhibiting *aflR* expression and also through inactivation of the *aflR* protein (Shimizu and Keller, 2001; Shimizu et al., 2003). Inhibition of FadA via FlbA allows asexual development and secondary metabolism production to occur (Hicks et al., 1997). This pathway was found conserved in other aspergilli; for instance, a constitutively activating mutation in FadA prevents AF production in *A. parasiticus* (Hicks et al., 1997).

#### VEA

The *veA* gene is well known as a regulator of light-dependent morphogenesis in aspergilli. *A. nidulans* cultures exposed to light develop asexually, forming abundant airborne conidia, while in the dark sexual development is favored, resulting in the formation of fruiting bodies called cleistothecia (Yager, 1992). Deletion of *veA* blocks cleistothecia production (Kim et al., 2002; reviewed in Calvo, 2008). It is also known that the *veA* gene plays a global regulatory role in the synthesis of secondary metabolites, including mycotoxins (Kato et al., 2003; Calvo et al., 2004; Cary et al., 2007; Duran et al., 2007; reviewed in Calvo, 2008). A study by Kato and colleagues (2003) showed that *veA* controls the transcription of genes necessary for the synthesis of the mycotoxin ST in *A. nidulans*. *veA* was shown to be required for the expression of *aflR* and subsequent activation of ST biosynthetic genes (Kato et al., 2003).

In addition, studies into the role of *veA* in the biosynthesis of AF in *A. parasiticus* and *A. flavus* revealed that *veA* was also necessary for *aflR* and *aflJ* transcription (Calvo et al., 2004; Cary et al., 2007; Duran et al., 2007). Furthermore, gene expression and chemical analyses showed that transcription of AF biosynthetic genes and AF production (Figure 3) are impaired in these mutants (Calvo et al., 2004; Duran et al., 2007). Additionally, the *A. flavus* and *A. parasiticus* deletion mutants were also unable to produce sclerotia. These findings, together with the fact that *veA* has only been found in fungi (unpublished phylogenetic studies in the Calvo laboratory), suggest that *veA* or *veA*-dependent genes could serve as targets for development of strategies to decrease the detrimental effects of mycotoxin contamination in our food supplies (Squire, 1981; Wu, 2006).

The *veA* gene also regulates the synthesis of additional secondary metabolites. For example, our studies showed that



**FIGURE 3** AFB1 analysis of *A. flavus* wild type and deletion *veA* mutant by thin-layer chromatography. 1–3 are replicates. AFB1 standard is shown on both sides.

the synthesis of cyclopiazonic acid and aflatrem was reduced or blocked in *A. flavus veA* deletion mutants (Duran et al., 2007). Cyclopiazonic acid (CPA), an indole tetramic acid, is toxic to animals and humans. CPA is a specific inhibitor of calcium-dependent ATPase in the sarcoplasmic reticulum, resulting in alteration of  $\text{Ca}^{++}$  levels, which leads to an increase in muscle contraction (Riley et al., 1992). The mycotoxin aflatrem, an indol-diterpene also produced by *A. flavus*, is a potent tremorgenic compound that causes neurological disorders (Valdes et al., 1985; Yao et al., 1989). *veA* is necessary for the expression of the aflatrem gene cluster (*atmG*, *atmC*, and *atmM*), explaining the absence of aflatrem production in this mutant (Duran et al., 2007).

Although the *veA* gene product does not present homology with any other proteins of known function, *veA* has been found in the genome of many filamentous fungi, being particularly conserved in *Ascomycetes* (Calvo et al., unpublished). The first study of *veA* orthologs in other fungal species beyond the genus *Aspergillus* was done in our laboratory on the plant pathogen *F. verticillioides*, a filamentous fungus that causes stalk and ear rot in corn (Li et al., 2006) and produces toxins called fumonisins (Gelderblom et al., 1988; Desai et al., 2002). Preliminary studies from the Calvo laboratory of *F. verticillioides* infecting natural plant substrates indicated that *FuVE1* is necessary for fumonisin production (Calvo, 2008) and pathogenicity (Calvo et al., unpublished). In addition, *veA* has been shown to regulate antibiotic production in different fungal genera, including *Aspergillus* (Kato et al., 2003; Dreyer et al., 2007).

As mentioned above, *veA* function is light-dependent. Numerous studies have used an *A. nidulans* mutant called *veA1* to study morphogenesis and regulation of secondary metabolism, including toxin production (Hicks et al., 1997; Shimizu and Keller, 2001). The *veA1* strains do not respond to light. The *veA1* mutant protein, lacking the first 36 amino acids, does not seem to prevent ST production; although ST production in *veA+* is greater than in *veA1* strains (Stinnett et al., 2007). Interestingly, in the same study, we showed that *A. nidulans veA* migration to the nucleus is light-dependent. While in the dark *veA* is located mainly in the nuclei, under light it is found abundantly in the cytoplasm. The *veA1* mutant protein lacks part of a functional bipartite nuclear localization signal present in the wild-type protein. The effect of light on ST production in a *veA* wild-type strain was also examined and found that the highest amount of mycotoxin was synthesized by the *veA+* strain cultured on glucose minimal medium in the dark, which favored the accumulation of *veA* protein in nuclei (Stinnett et al., 2007).

Blue light (440–500 nm) had a similar effect to that observed with white light, preventing an efficient accumulation of *veA* in the nuclei (Stinnett et al., 2007). The effect of exposure to red light (625–740 nm) was similar but more moderate in comparison with blue and white light. Blumenstein and colleagues (2005) reported that a phytochrome-like protein called FphA acts as a red-light sensor in *A. nidulans*. They demonstrated that FphA controlled morphogenesis. Previous studies suggested a possible interaction of *veA* with light-responsive proteins (Mooney and Yager, 1990; Stinnett et al., 2007) and it was recently demonstrated that FphA protein interacts with *veA* as part of a protein complex in the nucleus (Purschwitz et al., 2008). The *veA* complex also contained blue light-responsive proteins, LreA and LreB, orthologs to the respective *N. crassa* blue light-responsive CW1 and CW2 (Froehlich et al., 2002; He et al., 2002; Liu et al., 2003). LreA and LreB do not interact directly with *veA*, but through FphA interaction. FphA interacts with LreB, which was found to interact with LreA (Purschwitz et al., 2008; reviewed in Calvo, 2008). These proteins only interacted in the nucleus. FphA was shown to be a negative regulator of ST formation, whereas LreA and LreB had antagonistic roles with respect to FphA expression. These studies indicated that red- and blue-light perception occurs in

an integrative way in the *veA* protein complex affecting morphogenesis and secondary metabolism. Furthermore, *veA* abundance in the nucleus was negatively affected by FphA in the light. This, together with the fact that nuclear concentration of *veA* is also reduced by blue light (Stinnett et al., 2007), provide further evidence that both red- and blue-light sensing systems are influencing *veA* nuclear localization and consequently toxin production. The fact that the *veA* complex includes proteins that respond to external stimuli, such as light, and proteins that affect the expression of secondary metabolic gene clusters, such as *laeA*, further elaborated below (Bayram et al., 2008), suggest a possible scaffold role for *veA*. In this scaffold role, *veA* might integrate environmental cues, that is, light, through the interaction with light-sensing proteins (Purschwitz et al., 2008), with a genetic response by a *veA*-orchestrated action with other proteins (such as *laeA*, among others), leading to metabolic and developmental changes in an adaptive response (Calvo, 2008).

#### LAE A

The *laeA* gene was first discovered by complementation of an *A. nidulans* mutant strain unable to produce ST (Bok and Keller, 2004). *laeA* is a positive regulator of *aflR* expression, which in turn activates the expression of ST genes. As is the case in *veA* mutants, strains with a *laeA* deletion are defective in production of secondary metabolites (Bok and Keller, 2004; Hoffmeister and Keller, 2007; Perrin et al., 2007). The *laeA*-deduced amino acid sequence indicated that this protein could function as a methyltransferase, as it harbors a S-adenosyl methionine binding domain (Bok and Keller, 2004). ST cluster expression analysis showed that regulation by *laeA* is spatially limited to the cluster genes, not affecting genes adjacent to the cluster (Bok and Keller 2004; Bok, Noordermeer, et al., 2006; Perrin et al., 2007). The *A. nidulans laeA* microarray study by Bok et al. (Bok, Hoffmeister, et al., 2006) revealed a new secondary metabolite gene cluster regulated by *laeA*. This cluster was shown to be involved in the biosynthesis of the antitumoral compound terrequinone A (Bouhired et al., 2007). As in the case of the ST cluster, *laeA* regulation was restricted to the genes within the terrequinone A cluster.

The *laeA* gene has also been studied in the opportunistic mammalian pathogen *A. fumigatus*. The *laeA* deletion mutant was unable to produce gliotoxin among other compounds and presented a reduction in virulence in a murine model (Bok et al., 2005). The *laeA* mutant was also further characterized by functional genomics analysis (Perrin et al., 2007). Thirteen *A. fumigatus* gene clusters were found to be regulated by *laeA*, including those involved in the biosynthesis of gliotoxin and also pigments, fumitremorigens, festuclavine, elymoclavine, fumigaclavines, and ergot alkaloids.

The mechanism by which *laeA* regulates secondary metabolism is still unknown. Based on preliminary evidence, it is hypothesized that *laeA* may influence chromatin structure at cluster loci, thus controlling transcriptional activation of cluster genes (Keller et al., 2005; Bok, Noordermeer, et al., 2006). As mentioned above, the fact that *veA* and *laeA* proteins interact (Bayram et al., 2008) adds another layer of complexity to this regulatory mechanism, where this protein complex plays an important role in connecting external stimuli and adaptive genetic nuclear responses, including toxin production.

### **The Effect of Histone Acetylation on Toxin Biosynthesis**

The frequent cluster organization of genes involved in production of toxins and other secondary metabolites (Trail et al., 1995; Bradshaw et al., 2002; Walton, 2006) could present an evolutionary advantage for the organism. This could be due, at least in part, to the benefits of efficient global regulatory mechanisms influencing chromosomal organization. Roze and colleagues (Daniel et al., 1998; Roze, Miller, et al., 2004) identified a CRE1 binding protein (CRE1bp), responsive to cAMP levels, that has also been described as interacting with AfIR. Several CRE1-like binding sites are found in different AF gene promoters, and the interaction with CRE1bp has been confirmed in cases such as that of the *nor1* gene promoter. Importantly, CRE1 has been shown to recruit histone acetyltransferase (HAT) to promoter regions, leading to acetylation of histones, in particular histone H4, which has been demonstrated to enhance transcriptional activation (Daniel et al., 1998; Spencer and Davie, 1999; Shahbazian and Grunstein, 2007). In a recent study, Roze and colleagues (2007) demonstrated a

positive correlation between the initiation and spread of histone H4 acetylation in AF promoters, leading to the establishment of AF pathway gene expression and AF accumulation. This study revealed that a specific order of transcriptional activation of the *pksA*, *ver-1* and *omtA* genes followed a parallel pattern of histone H4 acetylation. The model postulates that declining glucose/sucrose levels in the growth medium are sensed by a G-protein coupled receptor, which transduces a signal through the cAMP/PKA pathway. This leads to dephosphorylation-dependent activation and binding of CRE1bp, which then recruits HAT, initiating the bidirectional wave (or waves) of histone H4 acetylation. AF gene transcription is enhanced by *aflR* binding to the AF promoter regions as their access is made available by changes in chromatin conformation. Previous studies have shown that when AF gene promoter-reporter fusions were integrated outside of the AF cluster, promoter activity was greatly decreased, supporting the model of specific regulation of cluster expression (Liang et al., 1997; Chiou et al., 2002). The role of epigenetic regulation of secondary metabolic gene clusters was further supported by recent evidence that deletion of the histone deacetylase (HDAC) gene, *hdaA*, in *A. nidulans* resulted in activation of transcription of genes of the ST and penicillin biosynthetic gene clusters (Shwab et al., 2007). Interestingly, while they showed that ST and penicillin production could be restored in a  $\Delta laeA \Delta hdaA$  double mutant, levels of these metabolites were not as high as in the  $\Delta hdaA$  alone. This suggests that *laeA* and *hdaA* operate through different mechanisms.

## Conclusion

In this review we have strived to present the current status of the literature on the molecular regulation of mycotoxin production in aspergilli with particular emphasis on the regulation of aflatoxin and sterigmatocystin production in *A. flavus* and *A. nidulans*, respectively. What began as a search for molecular mechanisms controlling the production of aflatoxin biosynthesis in *A. flavus* has now expanded to cover factors involved in the molecular regulation of fungal secondary metabolites in general as well as fungal development. Identification of the global regulators *LaeA* and *VeA*, in conjunction with an ever-increasing

database of whole genome sequence information from filamentous fungi, will almost certainly provide the basic tools necessary to develop practical and effective strategies to control production of mycotoxins that negatively impact both plants and mammals.

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